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**L-Lysine-producing corynebacteria and process for the  
preparation of lysine**

**Abstract**

5

The invention relates to L-lysine-producing strains of corynebacteria with amplified pyc gene (pyruvate carboxylase gene), in which strains additional genes, chosen from the group comprising the dapA gene 10 (dihydrodipicolinate synthase gene), the lysC gene (aspartate kinase gene), the lysE gene (lysine export carrier gene) and the dapB gene (dihydrodipicolinate reductase gene), but especially the dapA gene, are amplified and, in particular, overexpressed, and to a 15 process for the preparation of L-lysine.

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## ORIGINAL

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Invention Title:	L-lysine-producing Corynebacteria and Process for the Preparation of Lysine

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

**L-Lysine-producing corynebacteria and process for the preparation of lysine**

The invention relates to L-lysine-producing strains of 5 corynebacteria with amplified pyc gene (pyruvate carboxylase gene), in which strains additional genes, chosen from the group comprising the dapA gene (dihydrodipicolinate synthase gene), the lysC gene (aspartate kinase gene), the lysE gene (lysine export 10 carrier gene) and the dapB gene (dihydrodipicolinate reductase gene), but especially the dapA gene, are amplified and, in particular, overexpressed, and to a process for the preparation of L-lysine.

15 **State of the art**

L-Lysine is a commercially important L-amino acid which is used especially as a feed additive in animal nutrition. The need has been steadily increasing in recent years.

20 L-Lysine is prepared by a fermentation process with L-lysine-producing strains of corynebacteria, especially *Corynebacterium glutamicum*. Because of the great importance of this product, attempts are constantly being 25 made to improve the preparative process. Improvements to the process may relate to measures involving the fermentation technology, e.g. stirring and oxygen supply, or the composition of the nutrient media, e.g. the sugar concentration during fermentation, or the work-up to the 30 product form, e.g. by ion exchange chromatography, or the intrinsic productivity characteristics of the microorganism itself.

The productivity characteristics of these microorganisms are improved by using methods of mutagenesis, selection and mutant choice to give strains which are resistant to antimetabolites, e.g. S-(2-aminoethyl)cysteine, or-

5 auxotrophic for amino acids, e.g. L-leucine, and produce L-lysine.

Methods of recombinant DNA technology have also been used for some years in order to improve L-lysine-producing

10 strains of *Corynebacterium glutamicum* by amplifying individual biosynthesis genes and studying the effect on L-lysine production.

Thus EP-A-0 088 166 reports the increase in productivity,

15 after amplification, of a DNA fragment conferring resistance to aminoethylcysteine. EP-B-0 387 527 reports the increase in productivity, after amplification, of an *lysC* allele coding for a feedback-resistant aspartate kinase. EP-B-0 197 335 reports the increase in

20 productivity, after amplification, of the *dapA* gene coding for dihydridipicolinate synthase. EP-A-0 219 027 reports the increase in productivity, after amplification, of the *asd* gene coding for aspartate semialdehyde dehydrogenase. Pisabarro et al. (Journal of Bacteriology 175(9), 2743-

25 2749 (1993)) describe the *dapB* gene coding for dihydridipicolinate reductase.

The effect of the amplification of primary metabolism genes on L-lysine production has also been studied. Thus

30 EP-A-0 219 027 reports the increase in productivity, after amplification, of the *aspC* gene coding for aspartate aminotransferase. EP-B-0 143 195 and EP-B-0 358 940 report the increase in productivity, after amplification, of the *ppc* gene coding for phosphoenolpyruvate

carboxylase. DE-A-198 31 609 reports the increase in productivity, after amplification, of the pyc gene coding for pyruvate carboxylase. The anaplerotic reaction catalyzed by pyruvate carboxylase is of particular-  
5 importance compared with the reaction catalyzed by phosphoenolpyruvate carboxylase. Thus Wendisch et al. (FEMS Microbiology Letters 112, 269-274 (1993)) showed that the lysine production of the strain MH20-22B was not impaired by turning off the ppc gene.

10

Finally, Offenlegungsschrift DE-A-195 48 222 describes that an increased activity of the L-lysine export carrier coded for by the lysE gene promotes lysine production.

15 In addition to these attempts to amplify an individual gene, attempts have also been made to amplify two or more genes simultaneously and thereby to improve L-lysine production in corynebacteria. Thus Offenlegungsschrift DE-A-38 23 451 reports the increase in productivity, after  
20 simultaneous amplification, of the asd gene and the dapA gene from Escherichia coli. Offenlegungsschrift DE-A-39 43 117 discloses the increase in productivity, after simultaneous amplification, of an lysC allele coding for a feedback-resistant aspartate kinase and of the dapA gene.  
25 EP-A-0 841 395 particularly reports the increase in productivity, after simultaneous amplification, of an lysC allele coding for a feedback-resistant aspartate kinase and of the dapB gene; further improvements could be achieved by additional amplification of the dapB, lysA and  
30 ddh genes. EP-A-0 854 189 describes the increase in productivity, after simultaneous amplification, of an lysC allele coding for a feedback-resistant aspartate kinase and of the dapA, dapB, lysA and aspC genes. EP-A-0 857 784 particularly reports the increase in productivity, after

simultaneous amplification, of an *lysC* allele coding for a feedback-resistant enzyme of the *lysA* gene; a further improvement could be achieved by additional amplification of the *ppc* gene.

5

It is clear from the many processes described in the state of the art that there is a need for the development of novel approaches and for the improvement of existing processes for lysine production with corynebacteria.

10

#### Object of the invention

The object which the inventors set themselves was to provide novel L-lysine-producing strains of corynebacteria and processes for the preparation of L-lysine.

15

#### Description of the invention

L-Lysine is a commercially important L-amino acid which is used especially as a feed additive in animal nutrition.

When L-lysine or lysine is mentioned in the following text, it is understood as meaning not only the base but also the appropriate salts, e.g. lysine hydrochloride or lysine sulfate.

The invention provides L-lysine-producing strains of corynebacteria with amplified *pyc* gene (pyruvate carboxylase gene), wherein additional genes, chosen from the group comprising the *dapA* gene (dihydridopicolinate synthase gene), the *lysC* gene (aspartate kinase gene), the *lysE* gene (lysine export carrier gene) and the *dapB* gene (dihydridopicolinate reductase gene), but especially the

dapA gene, are amplified and, in particular, overexpressed.

5 The novel DNA sequence located upstream (5' end) from the dapB gene has also been found which carries the -35 region of the dapB promoter and is advantageous for the expression of the dapB gene. It is shown as SEQ ID No. 1.

10 A corresponding DNA capable of replication, with the nucleotide sequence shown in SEQ ID No. 1, is therefore claimed as well.

15 The invention also provides the MC20 and MA16 mutations of the dapA promoter shown in SEQ ID No. 5 and SEQ ID No. 6, deposited in the strains DSM12868 and DSM12867.

20 The invention also provides L-lysine-producing strains of corynebacteria with amplified pyc gene, wherein additionally the dapA and dapB genes are simultaneously amplified and, in particular, overexpressed.

25 The invention also provides L-lysine-producing strains of corynebacteria with amplified pyc gene, wherein additionally the dapA, dapB and lysE genes are simultaneously amplified and, in particular, overexpressed.

30 In this context the term "amplification" describes the increase in the intracellular activity, in a microorganism, of one or more enzymes which are coded for by the appropriate DNA, by increasing the copy number of the gene(s), using a strong promoter or using a gene coding for an appropriate enzyme with a high activity, and optionally combining these measures.

The invention also provides a process for the preparation of L-lysine using the bacteria described above.

- 5 The microorganisms which the present invention provides can prepare L-lysine from glucose, sucrose, lactose, fructose, maltose, molasses, starch or cellulose or from glycerol and ethanol, especially from glucose or sucrose. Said microorganisms are corynebacteria, especially of the
- 10 genus *Corynebacterium*. The species *Corynebacterium glutamicum* may be mentioned in particular in the genus *Corynebacterium*, being known to those skilled in the art for its ability to produce amino acids. This species includes wild-type strains such as *Corynebacterium glutamicum* ATCC13032, *Brevibacterium flavum* ATCC14067, *Corynebacterium melassecola* ATCC17965 and strains or mutants derived therefrom. Examples of L-lysine-producing mutants of corynebacteria are for example:
- 15
- 20 *Corynebacterium glutamicum* FERM-P 1709  
*Brevibacterium flavum* FERM-P 1708  
*Brevibacterium lactofermentum* FERM-P 1712  
*Brevibacterium flavum* FERM-P 6463  
*Brevibacterium flavum* FERM-P 6464
- 25 *Corynebacterium glutamicum* DSM5714  
*Corynebacterium glutamicum* DSM12866

The inventors have now found that an amplified expression of the *lysE* gene in addition to the *pyc* gene, or an

- 30 additionally amplified expression of an *lysC* allele coding for a feedback-resistant aspartate kinase, or an additionally amplified expression of the *dapB* gene and, in particular, an additionally amplified expression of the

dapA gene, individually or together, further improve L-lysine production.

5 It has also been found that, for a given overexpression of the pyc gene, the simultaneous, additionally amplified expression of the dapA and dapB genes brings further advantages for L-lysine production.

10 Finally, the inventors have also found that, for a given overexpression of the pyc gene, the simultaneous, additionally amplified expression of the dapA, dapB and lysE genes is extremely advantageous for L-lysine production.

15 An amplification (overexpression) is achieved e.g. by increasing the copy number of the appropriate genes or mutating the promoter and regulatory region or the ribosome binding site located upstream from the structural gene. Expression cassettes incorporated upstream from the 20 structural gene work in the same way. Inducible promoters additionally make it possible to increase the expression in the course of the formation of L-lysine by fermentation. Measures for prolonging the life of the mRNA also improve the expression. Furthermore, the enzyme 25 activity is also enhanced by preventing the degradation of the enzyme protein, the genes or gene constructs either being located in plasmids (shuttle vectors) of variable copy number or being integrated and amplified in the chromosome. Alternatively, it is also possible to achieve 30 an overexpression of the genes in question by changing the composition of the media and the culture technique.

Those skilled in the art will find relevant instructions inter alia in Martin et al. (Bio/Technology 5, 137-146

(1987)), Guerrero et al. (Gene 138, 35-41 (1994)),  
Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)),  
Eikmanns et al. (Gene 102, 93-98 (1991)), EP-0 472 869, US  
4,601,893, Schwarzer and Pühler (Bio/Technology 9, -84-87  
5 (1991)), Reinscheid et al. (Applied and Environmental  
Microbiology 60, 126-132 (1994)), LaBarre et al. (Journal  
of Bacteriology 175, 1001-1007 (1993)), patent application  
WO 96/15246, Malumbres et al. (Gene 134, 15-24 (1993)),  
Japanese Offenlegungsschrift JP-A-10-229891, Jensen and  
10 Hammer (Biotechnology and Bioengineering 58, 191-195  
(1998)) or the handbook "Manual of Methods for General  
Bacteriology" of the American Society for Bacteriology  
(Washington DC, USA, 1981) and well-known textbooks on  
genetics and molecular biology.

15 The genes from *Corynebacterium glutamicum* used according  
to the invention are described and can be isolated,  
prepared or synthesized by known methods.

20 Methods of localized mutagenesis are described inter alia  
by Higuchi et al. (Nucleic Acids Research 16, 7351-7367  
(1988)) or by Silver et al. in the handbook by Innis,  
Glefand and Sninsky (eds.) entitled PCR Strategies  
(Academic Press, London, UK, 1995).

25 The first step in isolating a gene of interest from *C.*  
*glutamicum* is to construct a gene library of this  
microorganism in e.g. *E. coli* or optionally also in *C.*  
*glutamicum*. The construction of gene libraries is  
30 documented in generally well-known textbooks and  
handbooks. Examples which may be mentioned are the  
textbook by Winnacker entitled From Genes to Clones,  
Introduction to Gene Technology (Verlag Chemie, Weinheim,  
Germany, 1990) or the handbook by Sambrook et al. entitled

Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). Bathe et al. (Molecular and General Genetics, 252: 255-265 (1996)) describe a gene library of *C. glutamicum* ATCC13032 which was constructed 5 using cosmid vector SuperCos I (Wahl et al., Proceedings of the National Academy of Sciences USA 84, 2160-2164 (1987)) in *E. coli* K-12 NM554 (Raleigh et al., Nucleic Acids Research 16: 1563-1575 (1988)). Börmann et al. (Molecular Microbiology 6(3), 317-326) in turn describe a 10 gene library of *C. glutamicum* ATCC13032 using cosmid pHG79 (Hohn and Collins, Gene 11, 291-298 (1980)). A gene library of *C. glutamicum* in *E. coli* can also be constructed using plasmids like pBR322 (Bolivar, Life Sciences 25, 807-818 (1979)) or pUC19 (Norlander et al., 15 Gene 26: 101-106 (1983)). In the same way it is also possible to use shuttle vectors such as pJC1 (Cremer et al., Molecular and General Genetics 220, 478-480 (1990)) or pEC5 (Eikmanns et al., Gene 102, 93-98 (1991)), which replicate in *E. coli* and *C. glutamicum*. Restriction- 20 and/or recombination-defective strains are particularly suitable hosts, an example being the *E. coli* strain DH5 $\alpha$ mcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA 87, 4645-4649 (1990)). Other examples are the restriction- 25 defective *C. glutamicum* strains RM3 and RM4, which are described by Schäfer et al. (Applied and Environmental Microbiology 60(2), 756-759 (1994)).

The gene library is then transferred to an indicator 30 strain by transformation (Hanahan, Journal of Molecular Biology 166, 557-580 (1983)) or electroporation (Tauch et al., FEMS Microbiological Letters, 123: 343-347 (1994)). The characteristic feature of the indicator strain is that it possesses a mutation in the gene of interest which

causes a detectable phenotype, e.g. an auxotrophy. The indicator strains or mutants are obtainable from publicized sources or strain collections, e.g. the Genetic Stock Center of Yale University (New Haven, Connecticut, USA), or if necessary are specially prepared. An example of such an indicator strain which may be mentioned is the *E. coli* strain RDA8 requiring mesodiaminopimelic acid (Richaud et al., C.R. Acad. Sci. Paris Ser. III 293: 507-512 (1981)), which carries a mutation (dapA::Mu) in the dapA gene.

After transformation of the indicator strain with a recombinant plasmid carrying the gene of interest, and expression of the gene in question, the indicator strain becomes prototrophic in respect of the appropriate characteristic. If the cloned DNA fragment confers resistance, e.g. to an antimetabolite like S-(2-aminoethyl)cysteine, the indicator strain carrying the recombinant plasmid can be identified by selection on appropriately supplemented nutrient media.

If the nucleotide sequence of the gene region of interest is known or obtainable from a data bank, the chromosomal DNA can be isolated by known methods, e.g. as described by Eikmanns et al. (Microbiology 140, 1817-1828 (1994)), and the gene in question can be synthesized by the polymerase chain reaction (PCR) using suitable primers and cloned into a suitable plasmid vector, e.g. pCRII TOPO from Invitrogen (Groningen, The Netherlands). A summary of PCR methodology can be found in the book by Newton and Graham entitled PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

Examples of publicly accessible data banks for nucleotide sequences are that of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany) or that of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

5 The isolation and cloning of the pyc gene from *C. glutamicum* ATCC13032 are described in DE-A-198 31 609 and by Koffas et al. (Applied Microbiology and Biotechnology 10 50, 346-352 (1988)). The nucleotide sequence of the pyc gene is obtainable under accession number AF038548 or Y09548.

15 The isolation and cloning of the lysE gene from *C. glutamicum* ATCC13032 are described in Offenlegungsschrift DE-A-195 48 222. The nucleotide sequence of the lysE gene is obtainable under accession number X96471.

20 The isolation, cloning and sequencing of the dapA gene from various strains of *C. glutamicum* are described by Cremer et al. (Molecular and General Genetics 220: 478-480 (1990)), by Pisabarro et al. (Journal of Bacteriology 175: 2743-2749 (1993)) and by Bonnassie et al. (Nucleic Acids Research 18: 6421 (1990)). DE-A-39 43 117 reports the 25 amplification of the dapA gene by means of plasmid pJC23. The nucleotide sequence of the dapA gene is obtainable under accession number X53993.

30 The isolation, cloning and sequencing of the dapB gene from *Brevibacterium lactofermentum* are described by Pisabarro et al. (Journal of Bacteriology 175: 2743-2749 (1993)). The nucleotide sequence of the dapB gene is obtainable under accession number X67737.

The isolation, cloning and sequencing of the lysC gene and of lysC alleles coding for a feedback-resistant aspartate kinase are reported by several autors (sic). Thus Kalinowski et al. (Molecular and General Genetics 224: 5 317-324 (1990)) report the lysC allele from the C. glutamicum strain DM58-1. DE-A-39 43 117 reports the cloning of the lysC allele from the C. glutamicum strain MH20. Follettie et al. (Journal of Bacteriology 175: 10 4096-4103 (1993)) report the lysC allele from the C. flavum strain N13, which is called ask in said publication. The nucleotide sequences of the lysC gene and of various lysC alleles are obtainable inter alia under accession numbers X57226 and E06826.

15 The genes obtained in this way can then be incorporated inter alia into plasmid vectors, e.g. pJC1 (Cremer et al., Molecular and General Genetics 220, 478-480 (1990)) or pEC5 (Eikmanns et al., Gene, 102, 93-98 (1991)), individually or in suitable combinations, transferred to 20 desired strains of corynebacteria, e.g. the strain MH20-22B (Schrumpf et al., Applied Microbiology and Biotechnology 37: 566-571 (1992)), by transformation, e.g. as in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), or by electroporation, 25 e.g. as in Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)), and expressed. The strain to be chosen can equally well be transformed with two plasmid vectors, each containing the gene or genes in question, thereby achieving the advantageous, simultaneously amplified 30 expression of two or more genes in addition to the known amplification of the pyc gene.

Examples of such strains are:

- the strain MH20-22B/pJC23/pEC7pyc, in which the pyc and dapA genes are expressed with simultaneous amplification, or

5   • the strain MH20-22B/pJC33/pEC7pyc, in which the pyc (sic) and the lysC(FBR) allele are simultaneously amplified and, in particular, overexpressed, or

10   • the strain MH20-22B/pJC23/pEC7dapBpyc, in which the pyc, dapA and dapB genes are simultaneously amplified and, in particular, overexpressed, or

15   • the strain MH20-22B/pJC23/pEC7lysEdapBpyc, in which the pyc, dapA, dapB and lysE genes are simultaneously amplified and, in particular, overexpressed.

The microorganisms prepared according to the invention can be cultivated for L-lysine production continuously or discontinuously by the batch process, the fed batch process or the repeated fed batch process. A summary of known cultivation methods is provided in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Bioprocess Technology 1. Introduction to Bioengineering) (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Bioreactors and Peripheral Equipment) (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

30   The culture medium to be used must appropriately meet the demands of the particular microorganisms. Descriptions of culture media for various microorganisms can be found in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C.,

USA, 1981). Carbon sources which can be used are sugars and carbohydrates, e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, e.g. soya oil, sunflower oil, groundnut oil and 5 coconut fat, fatty acids, e.g. palmitic acid, stearic acid and linoleic acid, alcohols, e.g. glycerol and ethanol, and organic acids, e.g. acetic acid. These substances can be used individually or as a mixture. Nitrogen sources which can be used are organic nitrogen-containing 10 compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used 15 individually or as a mixture. Phosphorus sources which can be used are potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium salts. The culture medium must also contain metal salts, e.g. magnesium sulfate or iron sulfate, which are 20 necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins can be used in addition to the substances mentioned above. Said feed materials can be added to the culture all at once or fed in appropriately during cultivation.

25

The pH of the culture is controlled by the appropriate use of basic compounds such as sodium hydroxide, potassium hydroxide or ammonia, or acid compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled using 30 antifoams such as fatty acid polyglycol esters. The stability of plasmids can be maintained by adding suitable selectively acting substances, e.g. antibiotics, to the medium. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gaseous mixtures, e.g. air,

into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until L-lysine formation has reached a maximum. This objective is normally achieved within 10 5 hours to 160 hours.

10 The concentration of L-lysine formed can be determined with the aid of amino acid analyzers by means of ion exchange chromatography and postcolumn reaction with ninhydrin detection, as described by Spackmann et al. (Analytical Chemistry 30, 1190 (1958)).

15 The following microorganisms have been deposited in the Deutsche Sammlung für Mikroorganismen und Zellkulturen 15 (German Collection of Microorganisms and Cell Cultures (DSMZ), Brunswick, Germany) under the terms of the Budapest Treaty:

- 20 • *Escherichia coli* K-12 strain DH5α/pEC7pyc as DSM12866
- *Escherichia coli* K-12 strain DH5α/pEC7dapBpyc as DSM12873
- 25 • *Escherichia coli* K-12 strain DH5α/pEC7lysEdapBpyc as DSM12874
- *Corynebacterium glutamicum* strain DSM5715/pJC23 as DSM12869
- 30 • *Corynebacterium glutamicum* strain DSM5715aecD::dapA(MA16) as DSM12867
- *Corynebacterium glutamicum* strain DSM5715aecD::dapA(MC20) as DSM12868
- 35 • *Corynebacterium glutamicum* strain DM678 as DSM12866

- *Escherichia coli* K-12 strain DH5 $\alpha$ /pEC7lysEpyc

### Examples

The present invention is illustrated in greater detail below with the aid of Examples.

5

#### Example 1

##### Preparation of the DNA coding for lysE

10 Chromosomal DNA was isolated from the strain ATCC13032 by the conventional methods (Eikmanns et al., *Microbiology* 140: 1817-1828 (1994)). The polymerase chain reaction (PCR) was used to amplify a DNA fragment carrying the lysE gene. The following primer oligonucleotides were chosen  
15 for the PCR on the basis of the lysE gene sequence known for *C. glutamicum* (Vrljic et al., *Molecular Microbiology* 22(5), 815-826 (1996)) (accession number X96471):

LysBam1:

20 5' CTC GAG AGC (GGA TCC) GCG CTG ACT CAC C 3'

LysBam2:

5' GGA GAG TAC GGC (GGA TCC) ACC GTG ACC 3'

25 The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press). The primers make it possible to amplify an approx. 1.1 kb DNA fragment carrying the lysE gene. The primers also  
30 contain the sequence for the cleavage site of the restriction endonuclease BamHI, which is indicated by brackets in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 1.1 kb, carrying the lysE gene, was identified by means of electrophoresis in 0.8% agarose gel, isolated from the gel and purified with the QIAquick Gel Extraction Kit (cat. no. 28704) from 5 Quiagen (sic) (Hilden, Germany).

The fragment was then ligated by means of T4 DNA ligase from Boehringer Mannheim (Mannheim, Germany) to vector pUC18 (Norrrander et al., Gene (26) 101-106 (1983)). This 10 was done by fully cleaving vector pUC18 with the restriction endonuclease SmaI and treating it with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany). The ligation mixture was transformed to the E. coli strain DH5 $\alpha$  (Hanahan, in: DNA cloning. A practical 15 approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Plasmid-carrying cells were selected by plating the transformation mixture on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) 20 which had been supplemented with 50 mg/l of ampicillin. Plasmid DNA was isolated from a transformant and checked by treatment with the restriction enzyme BamHI followed by agarose gel electrophoresis. The plasmid was called pUC18lysE.

25

#### Example 2

#### Preparation of dapB

30 Chromosomal DNA was isolated from the Corynebacterium glutamicum strain ATCC13032 as indicated in Example 1. The sequence of the dapB gene as such from Corynebacterium glutamicum is known (accession number X67737). However, the published DNA sequence comprises only 56 bp upstream

from the translation start, so the 5' end upstream from the translation start was additionally sequenced.

5 The sequencing was carried out with plasmid pJC25 (EP-B 0 435 132) using a primer oligonucleotide which binds in the region of the known dapB sequence (accession number X67737). The sequence of the sequencing primer used was: 5' GAA CGC CAA CCT TGA TTC C 3'

10 15 The sequencing was carried out by the chain termination method described by Sanger et al., Proc. Natl. Acad. Sci. USA, (74) 5463-5467 (1977). The sequencing reaction was performed with the aid of the AutoRead Sequencing Kit (Pharmacia, Freiburg). The electrophoretic analysis and detection of the sequencing products were carried out with the A.L.F. DNA sequencer from Pharmacia (Freiburg, Germany).

20 The DNA sequence obtained was used to choose a second primer in order to obtain further sequence data upstream from the transcription start. The following primer was chosen for this purpose:  
5' CTT TGC CGC CGT TGG GTT C 5' (sic)

25 The sequencing reaction was carried out as described above. The novel sequence upstream from the dapB gene is shown as SEQ ID No. 1. The sequence including the nucleotide sequence of the dapB gene is shown as SEQ ID No. 2.

30 The polymerase chain reaction was used to amplify the dapB gene. For this purpose, two primer oligonucleotides, chosen on the basis of the known DNA sequence of the dapB gene, were synthesized by MWG Biotech:

P-dap:

5' (AAG CTT) AGG TTG TAG GCG TTG AGC 3'

dapall:

5 5' TTA ACT TGT TCG GCC ACA GC 3'

The 5' primer (primer P-dap) contains a HindIII cleavage site which is indicated by brackets in the sequence shown above. The PCR was carried out as in Example 1. An 10 approx. 1.1 kb DNA fragment, which carries the dapB gene and contains a cleavage site for the restriction endonuclease HindIII at each end, was amplified in this way. The PCR fragment obtained was purified from 0.8% agarose gel (QIAquick Gel Extraction Kit from Qiagen, 15 Hilden, Germany) and cloned into cloning vector pCR2.1TOPO (Invitrogen, Leek, The Netherlands) with the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands, cat. no. K4550-01). The ligation mixture was transformed to the E. coli strain TOP10F' from Invitrogen, the transformation 20 mixture was plated on LB agar containing kanamycin (50 mg/l), IPTG (0.16 mM) and X-Gal (64 mg/l) and kanamycin-resistant, white colonies were isolated. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by cleavage with 25 the restriction enzyme HindIII followed by agarose gel electrophoresis. The DNA sequence of the amplified DNA fragment was checked by sequencing. The sequence of the PCR product matches the sequence shown in SEQ ID No. 1. The plasmid obtained was called pCR2.1TOPOdapB.

## Example 3

## Preparation of the DNA coding for pyc

5 The *Corynebacterium glutamicum* strain ATCC13032 was used as the donor for the chromosomal DNA. Chromosomal DNA was isolated from the strain ATCC13032 as described in Example 1. The polymerase chain reaction was used to amplify a DNA fragment carrying the pyc gene. The following primer oligonucleotides were chosen for the PCR on the basis of the pyc gene sequence known for *C. glutamicum* (Peters-Wendisch et al., *Microbiology* 144, 915-927 (1998)) (accession number Y09548):

10 15 5-PYC-IN:  
5' GC(T CTA GA)A GTG TCG CAA CCG TGC TTG A 3'  
3-PYC-IN:  
5' GC(T CTA GA)T TGA GCC TTG GTC TCC ATC T 3'

20 25 The primers shown were synthesized by MWG Biotech and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press). The primers make it possible to amplify an approx. 3.8 kb DNA fragment carrying the pyc gene. The primers also contain the sequence for a cleavage site of the restriction endonuclease XbaI, which is indicated by brackets in the nucleotide sequence shown above.

30 The amplified DNA fragment of approx. 3.8 kb, carrying the pyc gene, was identified by gel electrophoresis in 0.8% agarose gel, isolated from the gel and purified by the conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The fragment was then ligated to vector pCRII-TOPO by means of the Dual Promotor Topo TA Cloning Kit (Invitrogen, Leek, The Netherlands, cat. number K4600-01).

5 The ligation mixture was transformed to the *E. coli* strain TOP10 (Invitrogen, Leek, The Netherlands). Plasmid-carrying cells were selected by plating the transformation mixture on LB agar containing kanamycin (50 mg/l) and X-Gal (64 mg/l).

10

After isolation of the DNA, the plasmid obtained was checked by means of restriction cleavage and identified in agarose gel. The plasmid was called pCRII-TOPOpyc and the DNA sequence of the cloned insert was sequenced for 15 control purposes. As the determined sequence of the pyc insert in pCRII-TOPOpyc matches the sequence of the gene library entry, this plasmid was used subsequently.

#### Example 4

20

#### Cloning of dapB into vector pEC7

An approx. 1.1 kb DNA fragment carrying the dapB gene was isolated from plasmid pCR2.1TOPOdapB (from Example 2).

25 For this purpose, plasmid pCR2.1TOPOdapB was fully digested with the restriction enzyme HindIII and the approx. 1.1 kb DNA fragment carrying the dapB gene was isolated.

30 The dapB fragment was inserted into vector pEC7. Vector pEC7 is based on *E. coli* - *C. glutamicum* shuttle vector pEC5 (Eikmanns et al., 102: 93-98 (1991)). The BamHI cleavage site not located in the polylinker was removed from plasmid pEC5 in the following manner: Plasmid pEC5

was partially cleaved with the restriction enzyme BamHI. The approx. 7.2 kb DNA fragment was isolated from the agarose gel and the protruding ends were filled in with Klenow polymerase (Boehringer Mannheim). The resulting 5 DNA fragment was ligated (T4 ligase, Boehringer Mannheim). The ligation mixture was transformed to the E. coli strain DH5 $\alpha$  and kanamycin-resistant colonies were isolated on LB agar containing kanamycin (50 mg/l). Plasmid DNA was isolated from a transformant (QIAprep Spin Miniprep Kit 10 from Qiagen) and checked by restriction cleavage with the restriction enzymes BamHI and PstI. The resulting plasmid was called pEC6.

Plasmid pEC6 was fully cleaved with the restriction enzyme 15 XhoI. A DNA fragment carrying the trp terminator was ligated to vector DNA fragment (T4 ligase, Boehringer Mannheim). The ligation mixture was transformed to the E. coli strain DH5 $\alpha$  and kanamycin-resistant colonies were isolated on LB agar containing kanamycin (50 mg/l).

20 Plasmid DNA was isolated from a transformant (QIAprep Spin Miniprep Kit from Qiagen) and checked by restriction cleavage with the restriction enzymes BamHI and XhoI. The resulting plasmid was called pEC7.

25 The dapB-carrying DNA fragment obtained was ligated to vector pEC7 (T4 DNA ligase, Boehringer Mannheim), which had also been fully digested with the restriction enzyme HindIII and treated with alkaline phosphatase (Boehringer Mannheim). The ligation mixture was transformed to the E. 30 coli strain DH5 $\alpha$  and kanamycin-resistant colonies were isolated on LB agar containing kanamycin (50 mg/l). Plasmid DNA was isolated from a transformant (QIAprep Spin Miniprep Kit from Qiagen) and checked by restriction

cleavage with the restriction enzyme HindIII. The resulting plasmid was called pEC7dapB (Figure 1). The *Escherichia coli* strain obtained was called DH5 $\alpha$ /pEC7dapB.

5 Example 5

Cloning of lysE into vector pEC7

Plasmid pUC18lysEneu described in Example 1 was fully  
10 digested with the restriction enzyme BamHI and the 1.1 kb  
BamHI fragment carrying the lysE gene was isolated as in  
Example 1. Vector pEC7 was likewise fully cleaved with  
the restriction enzyme BamHI and treated with alkaline  
phosphatase. The BamHI vector fragment and the BamHI lysE  
15 fragment were ligated (Rapid DNA Ligation Kit, Boehringer  
Mannheim) and transformed to the *E. coli* strain DH5 $\alpha$ .  
Plasmid-carrying transformants were selected on LB agar  
containing chloramphenicol (10 mg/l). Plasmid DNA was  
isolated (QIAprep Spin Miniprep Kit, Qiagen) and checked  
20 by restriction cleavage with the enzyme BamHI. The  
resulting plasmid was called pEC7lysE (Figure 2). The  
strain obtained by transformation of plasmid pEC7lysE to  
the *E. coli* strain DH5 $\alpha$  was called DH5 $\alpha$ /pEC7lysE.

25 Example 6

Cloning of pyc into vector pEC7

The 3.8 kb DNA fragment carrying the pyc gene from *C. glutamicum* ATCC13032 was obtained from plasmid pCRII-  
30 TOP0pyc (from Example 3) by cleavage with the restriction enzyme XbaI. The 3.8 kb DNA fragment was identified by  
gel electrophoresis, isolated from the gel and purified by  
the conventional methods and the protruding ends were

filled in with Klenow polymerase. Vector pEC7 was likewise fully cleaved with the restriction enzyme SmaI and treated with alkaline phosphatase. The SmaI vector fragment and the XbaI pyc fragment treated with Klenow 5 polymerase were ligated (T4 ligase, Boehringer Mannheim) and transformed to the *E. coli* strain DH5 $\alpha$ . Plasmid-carrying transformants were selected on LB agar containing chloramphenicol (10 mg/l). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and 10 checked by restriction cleavage with the restriction enzyme SalI. The resulting plasmid was called pEC7pyc (Figure 3). The *E. coli* strain obtained by transformation of plasmid pEC7pyc to the *E. coli* strain DH5 $\alpha$  was called DH5 $\alpha$ /pEC7pyc.

15

#### Example 7

##### Preparation of a plasmid containing lysE and dapB

20 The dapB gene was isolated as a HindIII fragment from plasmid pCR2.1TOPOdapB containing the dapB gene from *C. glutamicum* ATCC13032. To do this, the plasmid was fully digested with the restriction enzyme HindIII and the dapB-carrying DNA fragment was isolated from 0.8% agarose gel 25 (QIAquick Gel Extraction Kit, Qiagen).

Vector pEC7lysE was also fully digested with the restriction enzyme HindIII and treated with alkaline phosphatase. The 1.1 kb fragment containing dapB was 30 ligated to the resulting linear vector fragment (T4 ligase, Boehringer Mannheim) and the ligation mixture was transformed to the *E. coli* strain DH5 $\alpha$ . Plasmid-carrying transformants were selected on LB agar containing

chloramphenicol (10 mg/l). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and checked by restriction cleavage with the restriction enzyme HindIII.

5

The resulting plasmid was called pEC7lysEdapB. This plasmid is capable of autonomous replication in *Escherichia coli* and in *Corynebacterium* and confers resistance to the antibiotic chloramphenicol on its host.

10

Plasmid pEC7lysEdapB simultaneously contains the dapB gene, which codes for dihydridipicolinate reductase, and the lysE gene, which codes for the lysine exporter.

15

The strain obtained by the transformation of *E. coli* DH5 $\alpha$  with pEC7lysEdapB was deposited.

#### Example 8

20

Preparation of a plasmid simultaneously containing dapB and pyc

The plasmid carrying the pyc gene which codes for the pyruvate carboxylase from *Corynebacterium glutamicum*

25

ATCC13032 was fully cleaved with the restriction enzyme XbaI and the protruding ends were filled in with Klenow polymerase as described in Example 6, making it possible to isolate the 3.8 kb DNA fragment containing the gene for pyruvate carboxylase.

30

Plasmid pEC7dapB (from Example 4) was also fully cleaved with the restriction enzyme SmaI and the ends were treated with alkaline phosphatase. The resulting linear vector fragment was ligated to the 3.8 kb DNA fragment containing

the pyc gene using T4 DNA ligase (Boehringer Mannheim, Mannheim, Germany). This produced a plasmid containing both the dapB gene and the pyc gene from corynebacteria. Plasmid-carrying transformants were selected on LB-agar 5 containing chloramphenicol (10 mg/l). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and verified by restriction cleavage with the restriction enzyme SalI. The plasmid is shown in Figure 4 and was called pEC7dapBpyc. The E. coli strain obtained 10 by transformation of plasmid pEC7dapBpyc to the E. coli strain DH5 $\alpha$  was called DH5 $\alpha$ /pEC7dapBpyc.

#### Example 9

15 Preparation of a plasmid containing sequences simultaneously coding for lysE, dapB and pyc

Plasmid pCRII-TOPOpyc (from Example 3), which carries the pyc gene coding for the pyruvate carboxylase from 20 Corynebacterium glutamicum ATCC13032, was fully cleaved with the restriction enzyme XbaI and treated with Klenow polymerase as described in Example 6, making it possible to isolate the 3.8 kb DNA fragment containing the gene for pyruvate carboxylase.

25 Plasmid pEC7dapBlysE was also fully cleaved with the restriction enzyme SmaI and the ends were treated with alkaline phosphatase. The resulting linear vector fragment was ligated to the 3.8 kb DNA fragment containing 30 the pyc gene using T4 DNA ligase (Boehringer Mannheim). This produces a plasmid containing both the lysE gene and dapB gene and the pyc gene from Corynebacterium glutamicum. Plasmid-carrying transformants were selected on LB agar containing chloramphenicol (10 mg/l). Plasmid

DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and verified by restriction cleavage with the restriction enzyme SmaI. The plasmid is shown in Figure 5 and was called pEC7dapBlysEpyc. The *E. coli* 5 strain obtained by transformation of plasmid pEC7dapBlysEpyc to the *E. coli* strain DH5 $\alpha$  was called DH5 $\alpha$ /pEC7dapBlysEpyc.

Example 10

10

Transformation of the strain MH20-22B with plasmids pJC23 and pJC33

Plasmid pJC1 is a plasmid capable of replication in 15 *Escherichia coli* and *Corynebacterium glutamicum* (Cremer et al., Molecular and General Genetics 220: 478-480 (1990)). Plasmid pJC33 (Cremer et al., Applied and Environmental Microbiology 57(6), 1746-1752 (1991)), which carries the lysC(Fbr) gene from the *C. glutamicum* strain MH20-22B, is 20 derived therefrom.

Plasmid pJC23 is also based on vector pJC1 and carries the dapA gene from *C. glutamicum* ATCC13032 (Cremer et al., Molecular and General Genetics 220: 478-480 (1990)) (EP-B 25 0 435 132). Plasmids pJC1, pJC33 and pJC23 were introduced into the strain MH20-22B by the electroporation method (Haynes and Britz, FEMS Microbiology Letters (61) 329-334 (1989)). The *C. glutamicum* strain MH20-22B is an AEC-resistant lysine producer deposited under the number 30 DSM5715.

The transformants obtained by means of electroporation were isolated on selection agar (LBHIS agar (18.5 g/l of brain-heart infusion broth, 0.5 M sorbitol, 5 g/l of bacto

tryptone, 2.5 g/l of bacto yeast extract, 5 g/l of NaCl, 18 g/l of bacto agar)) containing 15 mg/l of kanamycin. Plasmid DNA was isolated by the conventional methods (Peters-Wendisch et al., Microbiology 144, 915-927-5 (1998)), cleaved with suitable restriction endonucleases and checked. The strains obtained were called MH20-22B/pJC1, MH20-22B/pJC33 and MH20-22B/pJC23.

Example 11  
10 Transformation with plasmids pEC7pyc, pEC7dapBpyc and pEC7dapBlysEpyc

15 The strains prepared in Example 10 were subsequently provided with a second plasmid.

20 The following plasmids were introduced by the electroporation method into the strains MH20-22B/pJC1, MH20-22B/pJC33 and MH20-22B/pJC23 described:

- 25 • pEC7pyc (cf. Example 6)
- pEC7dapBpyc (cf. Example 7) (sic)
- pEC7dapBlysEpyc (cf. Example 8) (sic)

30 The transformed bacteria are selected on the basis of the antibiotic resistance of the plasmids they contain. The transformants obtained by means of electroporation were isolated on selection agar (LBHIS agar containing 15 mg/l of kanamycin and 7.5 mg/l of chloramphenicol). Plasmid DNA was isolated, cleaved with suitable restriction endonucleases and checked.

35 The strains obtained are listed below:

DSM5715/pJC1/pEC7pyc

DSM5715/pJC33/pEC7pyc

5

DSM5715/pJC23/pEC7pyc

DSM5715/pJC23/pEC7dapBpyc

10 DSM5715/pJC23/pEC7lysEdapBpyc

Example 12:

Preparation of L-lysine

15

The various *C. glutamicum* strains obtained in Examples 10 and 11 were cultivated in a nutrient medium suitable for lysine production and the lysine content of the culture supernatant was determined.

20

This was done by first incubating the various strains on agar plates with the appropriate antibiotics (brain-heart agar containing kanamycin (25 mg/l), chloramphenicol (10 mg/l)) for 24 h at 33°C. These agar plate cultures were

25

used to inoculate a preculture (10 ml of medium in a 100 ml conical flask). Complete medium CgIII was used as the preculture medium. Kanamycin (25 mg/l) and chloramphenicol (10 mg/l) were added. The preculture was incubated for 24 hours at 33°C on a shaker at 240 rpm.

30

This preculture was used to inoculate a main culture to give an initial OD (660 nm) of 0.2. Medium MM was used for the main culture.

Medium MM:

35

CSL 5 g/l

MOPS 20 g/l

	Glucose	50 g/l (autoclave separately)
Salts:		
5	$(\text{NH}_4)_2\text{SO}_4$	25 g/l
	$\text{KH}_2\text{PO}_4$	0.1 g/l
10	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.0 g/l
	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	10 mg/l
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	10 mg/l
15	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	5.0 mg/l
	Biotin	0.3 mg/l (sterile-filtered)
20	Thiamine*HCl	0.2 mg/l (sterile-filtered)
	$\text{CaCO}_3$	25 g/l

Abbreviations:

25	CSL:	corn steep liquor
	MOPS:	morpholinopropanesulfonic acid

CSL, MOPS and the salt solution are adjusted to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and 30 vitamin solutions and the dry-autoclaved  $\text{CaCO}_3$  are then added.

Cultivation is carried out in a volume of 10 ml in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) 35 and chloramphenicol (10 mg/l) were added. Cultivation proceeded at 33°C and 80% atmospheric humidity.

After 72 hours the OD was measured at a wavelength of 660 nm. The amount of lysine formed was determined with 40 an amino acid analyzer from Eppendorf BioTronik (Hamburg, Germany) by means of ion exchange chromatography and

postcolumn derivatization with ninhydrin detection. The glucose content was determined with a sugar analyzer from Skalar Analytik GmbH (Erkelenz, Germany).

5 The experimental results are shown in Table 1.

Table 1

Strain	Gene	OD (660)	Lysine-HCl g/l
DSM5715/pJC1/pEC7pyc	pyc	9.3	11.3
DSM5715/pJC33/pEC7pyc	pyc, lysC(Fbr)	9.2	11.9
DSM5715/pJC23/pEC7pyc	pyc, dapA	9.3	14.4
DSM5715/pJC23/pEC7dapBpyc	pyc, dapA, dapB	9.3	16.9
DSM5715/pJC23/ pEC7lysEdapBpyc	pyc, dapA, dapB, lysE	9.1	17.6

## SEQUENCE LISTING

5 <110> Degussa-Hüls AG  
Forschungszentrum Jülich GmbH

10 <120> L-Lysine-producing corynebacteria and  
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## Figures

The following Figures are attached:

5     • Figure 1: pEC7dapB (cf. Example 4)  
      • Figure 2: pEC7lysE (cf. Example 5)  
      • Figure 3: pEC7pyc (cf. Example 6)  
10     • Figure 4: pEC7dapBpyc (cf. Example 8)  
      • Figure 5: pEC7lysEdapBpyc (cf. Example 9)

15     The abbreviations used in the Figures are defined as follows:

          Cm:       chloramphenicol resistance gene  
20    dapB:       dapB gene from *C. glutamicum*  
          lysE:       lysE gene from *C. glutamicum*  
          pyc:       pyc gene from *C. glutamicum*  
25    OriE:       plasmid-coded origin of replication of *E. coli*  
          pBL:       DNA fragment of plasmid pBL1  
          EcoRI:      cleavage site of the restriction enzyme EcoRI  
          EcoRV:      cleavage site of the restriction enzyme EcoRV  
          HincII:     cleavage site of the restriction enzyme HincII  
35    HindIII:     cleavage site of the restriction enzyme HindIII  
          KpnI:       cleavage site of the restriction enzyme KpnI  
40    SalI:       cleavage site of the restriction enzyme SalI

SmaI: cleavage site of the restriction enzyme SmaI

SphI: cleavage site of the restriction enzyme SphI

5 PvuII: cleavage site of the restriction enzyme PvuII

BamHI: cleavage site of the restriction enzyme BamHI

**L-Lysine-producing corynebacteria and process for the preparation of lysine**

**CLAIMS:** The claims defining the invention are as follows:

- 5 1. L-Lysine-producing corynebacteria with an amplified pyc gene, in which additionally genes chosen from the group comprising the dapA gene, the lysC gene, the lysE gene and the dapB gene, individually or together, are amplified and, in particular, overexpressed.
- 10 2. Corynebacteria as claimed in claim 1 in which the dapA gene and optionally the dapB gene are amplified and, in particular, overexpressed.
- 15 3. Corynebacteria as claimed in claim 1 in which the dapA gene, the dapB gene and the lysE gene are amplified and, in particular, overexpressed.
- 20 4. Corynebacteria as claimed in claim 1 which contain the MC20 or MA16 mutations of the dapA promoter shown in SEQ ID No. 5 and SEQ ID No. 6.
- 25 5. Corynebacteria as claimed in claim 1 in which the dapB gene, which additionally contains the 5' end upstream from the translation start of this gene, shown in SEQ ID No. 1, is amplified and, in particular, overexpressed.
- 30 6. Preferably recombinant DNA originating from Corynebacterium and capable of replication in corynebacteria, which contains at least the nucleotide sequence additionally containing the 5'

end upstream from the translation region of the dapB gene, shown in SEQ ID No. 1.

7. DNA capable of replication, as claimed in claim 5, with the nucleotide sequence shown in SEQ ID No. 1.
8. A process for the preparation of L-lysine by the fermentation of corynebacteria with an amplified pyc gene, wherein bacteria are used in which nucleotide sequences coding for genes chosen from the group comprising dapA, lysC, lysE and dapB, individually or together, are amplified and, in particular, overexpressed.
- 15 9. The process as claimed in claim 8 wherein bacteria are used in which the dapA gene and optionally simultaneously the lysC gene are amplified and, in particular, overexpressed.
- 20 10. The process as claimed in claim 8 wherein bacteria are used in which the dapA gene, the dapB gene and the lysE gene are simultaneously amplified and, in particular, overexpressed.
- 25 11. The process as claimed in claim 8 wherein a strain transformed with one or more plasmid vectors is used, the plasmid vector(s) carrying the nucleotide sequences for one or more of the genes to be amplified.
- 30 12. The process as claimed in claim 8 wherein a strain transformed with one or more plasmid vectors is used and the plasmid vector carries the nucleotide sequences which code for one or more genes chosen

from the group comprising the pyc, dapA, dapB and/or lysE genes.

13. The process as claimed in one or more of the -  
5 preceding claims wherein bacteria of the species  
Corynebacterium glutamicum are used.

14. The process for the preparation of L-lysine by  
10 fermentation as claimed in one or more of the  
preceding claims wherein the following steps are  
carried out:

- a) fermentation of the L-lysine-producing bacteria  
in which the genes described are amplified,
- b) enrichment of L-lysine in the medium or in the  
15 cells of the bacteria, and
- c) isolation of the L-lysine.

15. Escherichia coli K-12 strain DH5 $\alpha$ /pEC7lysE, deposited  
as DSM12871.

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16. Escherichia coli K-12 strain DH5 $\alpha$ /pEC7dapBlysE,  
deposited as DSM12875.

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17. Corynebacterium glutamicum strain DSM5715/pJC23,  
deposited as DSM12869.

18. Corynebacterium glutamicum strain  
DSM5715aecD::dapA(MA16), deposited as DSM12867.

30

19. Corynebacterium glutamicum strain  
DSM5715aecD::dapA(MC20), deposited as DSM12868.

20. Corynebacterium glutamicum strain 678, deposited as  
DSM12866.

21. DNA capable of replication, with the nucleotide sequence MC20 shown in SEQ ID No. 5.
22. DNA capable of replication, with the nucleotide sequence MA16 shown in SEQ ID No. 6.
23. L-lysine-producing corynebacteria with an amplified pyc gene, substantially as hereinbefore described with reference to any one of the examples.
24. Preferably recombinant DNA originating from corynebacterium and capable of replication in corynebacteria, substantially as hereinbefore described with reference to any one of the examples.
- 10 25. A process for the preparation of L-lysine by the fermentation of corynebacteria with an amplified pyc gene, substantially as hereinbefore described with reference to any one of the examples.
26. The product of the process of any one of claims 8 to 14 or 25.
27. The use of L-lysine-producing corynebacteria of any one of claims 1 to 5 or 15 prepared by the process of any one of claims 8 to 14 or 25 for preparing L-lysine.

**Dated 7 June, 2000**

**Degussa-Huls Aktiengesellschaft  
Forschungszentrum Jülich GmbH**

**Patent Attorneys for the Applicant/Nominated Person**

**SPRUSON & FERGUSON**

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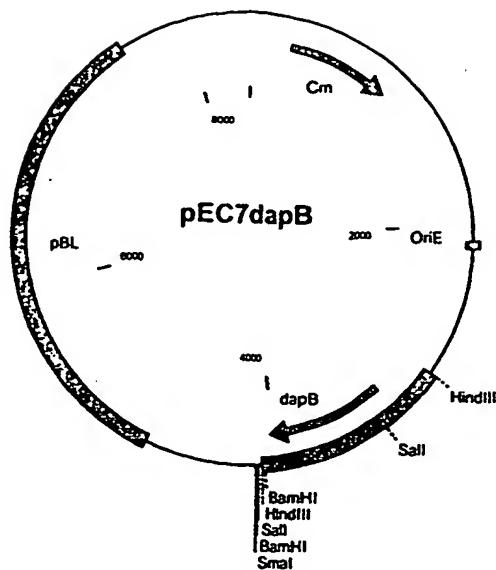


Figure 1: Plasmid pEC7dapB

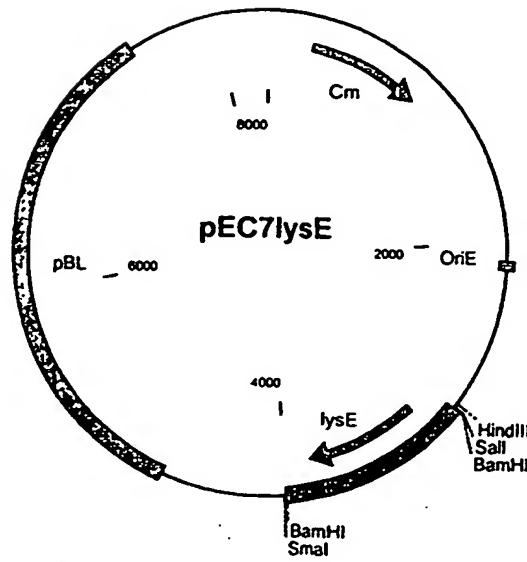


Figure 2: Plasmid pEC7lysE

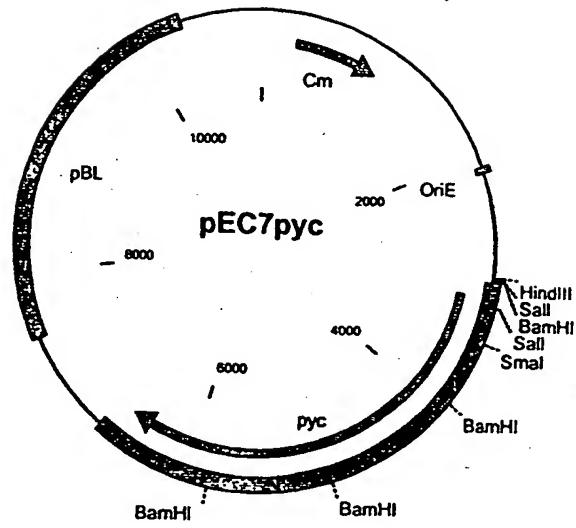


Figure 3: Plasmid pEC7pyc

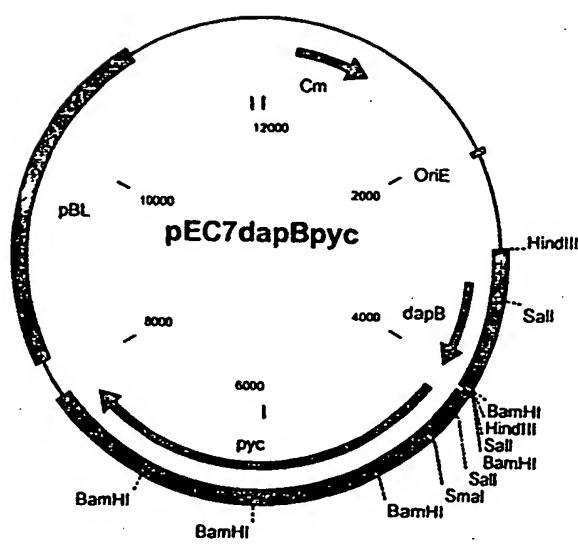


Figure 4: Plasmid pEC7dapBpyc

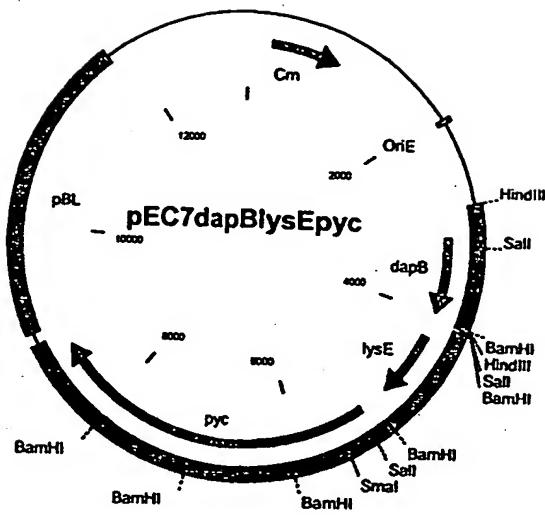


Figure 5: Plasmid pEC7dapBlysEpyc

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